New insights on stomata analysis of European conifers 65 years after the pioneering study of Werner Trautmann (1953)

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Abstract

Conifer-stomata analysis is an essential part of the palaeoecological toolbox because it allows determining the local presence of plant populations with a lower degree of uncertainty than pollen analysis. Although the European postglacial pool of conifer taxa is broad, stomata morphologies for only few taxa were investigated. Prior stomata morphology studies focused on taxa having wide distribution ranges in central and northern Europe, and stomata-morphologies for taxa occurring in southern European and Northern African mountain regions have not been described, yet.

Here, we present a qualitative assessment of stomata morphologies for 40 taxa from eight genera (*Cupressus*, *Juniperus*, *Abies*, *Cedrus*, *Larix*, *Picea*, *Pinus*, and *Taxus*) that are present on the European continent and the southern borderlands of the Mediterranean Basin, thereby broadening substantially both the regional and taxonomical coverage of this now 65-years old technique. We found that visual identification of conifer stomata does not allow species-level identifications, supporting the notion of genus-specific stomata morphologies found in prior studies. For each genus we describe the stomata morphologies taking into account the varying shape of stomata features at different focusing levels. In addition, we provide stop-motion animations (publicly available at http://dx.doi.org/10.6084/m9.figshare.7165261) that may be useful tools for microscope analysts who want to acquaint oneself with conifer-stomata analysis.

Keywords: stomata; conifers; vegetation history; Europe; reference-collection

Introduction

Natural ecosystems and biodiversity will be substantially affected by changes in climate and land-uses during the ongoing century (Alcamo et al. 2007) and endemic plants in biodiversity hotspots appear particularly vulnerable to environmental changes (Malcolm et al. 2006). Organisms may be at risk if their habitat is fragmented or lost, and if they will be unable to rapidly adjust to new environmental conditions (Parmesan 2006). The risk of species loss may be particularly high for species having small or isolated populations such as those living on islands or in so-called 'habitat islands' on the continents (Whittaker and Fernandez-Palacios 2007).

Among the species living in such 'habitat islands' are several conifer species. Due to their conservative traits (tough, long-lived needles; narrow tracheids enabling persistence in boreal regions), in Europe, Asia, and North America they are often restricted to high-latitudes, subalpine forests, arid regions, and on nutrient-poor or poorly drained soils, where they can escape or reduce the competitive effects of angiosperms (Coomes et al. 2005).

The Mediterranean Basin hosts among the conifers a variety of endemic species (and subspecies) having currently small natural populations located in mountain regions (Fig. 1). Several of these species are deemed to be vulnerable, endangered, or critically endangered, i.e. species considered to be facing a high, very high, or extremely high risk of extinction in the wild (IUCN 2017). However, because these assessments rely only on recent population-size estimates, little is known concerning longer-term changes of range size and populations. Thus, for several of these endemic conifer species the historical legacies and the impacts of past environmental changes (climate, land-use, fire disturbance) are not well constrained over longer time spans.

Past range shifts and population-size estimates are mostly inferred based on pollen records (e.g. Huntley and Birks 1983; Conedera et al. 2004; Giesecke et al. 2017; Brewer et al. 2017). However, particularly in mountain settings uncertainties related to pollen dispersal limit the possibility to infer the presence and history of parent trees around a study site (Herring et al. 2018). The palaeoecological toolbox offers, however, methods to prevail upon these limitations of pollen analysis: plant-macrofossil analysis (Birks and Birks 2000) and conifer-stomata analysis in pollen slides (Ammann et al. 2014) both can provide records at higher spatial resolution than pollen (Gervais and MacDonald 2001; Birks and Bjune 2010; Finsinger et al. 2017). However, conifer needles can be degraded after needle fall from parent trees and therefore be absent from plant-macrofossil records. By contrast, in such cases stomata can be found in pollen slides because their lignified components are more resistant than plant macrofossils to degradation in sedimentary archives, and chemicals used for pollen preparation do not degrade their lignified structures. Thus, pollen, plant macrofossils, and stomata provide

complementary evidences to unfold both altitudinal (Ammann et al. 2014; Vincze et al. 2017; Orbán et al. 2018), latitudinal range shifts (Froyd 2005; Wagner et al. 2015), or range-size contractions (Tinner et al. 2013).

In his pioneering work, the European botanist Werner Trautmann (1953) investigated the morphologies of stomata from central European trees and shrubs of six conifer genera (*Taxus baccata, Abies alba, Picea abies, Larix decidua, Pinus* with 4 species, and *Juniperus* with 3 taxa; see Table 1) thereby laying the foundation of sedimentary conifer-stomata analysis. He showed that stomata could be identified to genus level, an inference later confirmed by Sweeney (2004), who focused on the six conifer species present in Scandinavia during postglacial times (Table 1). Meanwhile, on the basis of Trautmann's pioneering work (Lang 1994) identification keys of conifer stomata have been developed for species from North America (Hansen 1995; Lacourse et al. 2016), South America (Hansen et al. 2003), and China (Hu et al. 2016), thereby extending the spatial coverage of conifer-stomata analysis to other continents and their floras.

While conifer-stomata analysis has increasingly been used and improved, the stomata morphologies of southern European and Mediterranean endemic conifers have not been described, yet. Here, we portray and compare the stomata morphologies for 40 conifer taxa that are native to the European continent and the southern borderlands of the Mediterranean Basin (Table 1). The aim is to provide a first qualitative assessment of their features that may be helpful to reconstruct past shifts in ranges and treelines in southern European and Mediterranean mountain regions and might be extended later by quantitative analyses. Our main reason to stay with qualitative analyses is that there is evidence that at continental scales some stomata measures (e.g. size) may vary among populations (García Álvarez et al. 2009). Thus spatially highly resolved continental efforts would be needed for quantitative analyses, which is out of scope for this study. To support our descriptions of stomata morphologies, we devised a method to illustrate the stomata morphologies with stop-motion animations. The animations offer stomata analysts the navigation across the different layers of the stomata structures, a mandatory procedure to produce reliable stomata identifications (Trautmann 1953). Based on the microscope analysis, we provide for each genus a qualitative description of features that we deem important to identify the stomata to the lowest possible taxonomical level on the basis of qualitative criteria.

Materials & Methods

Collection of needles

Conifer needles were collected from herbaria (the Herbier Montpellier Université (MPU; Montpellier, France) and the Royal Botanic Gardens Kew (KEW; London, UK)), and from living specimens growing at the Botanical Gardens of the Universities of Montpellier (Jardin des Plantes; Montpellier, France), Vienna (Hortus Botanicus Vindobonensis – HBV; Vienna, Austria), and Bern (BOGA; Bern, Switzerland), and from stands in Serbia and Romania (see ESM_1 at (available at http://dx.doi.org/10.6084/m9.figshare.7165261). Species names written on the nametags of collected specimens were crosschecked against the GBIF Backbone Taxonomy (GBIF Secretariat 2017) and synonyms were replaced with the currently accepted species (or subspecies) names.

Laboratory treatment

Needles of each specimen were first dried at 60ºC overnight and thereafter cut with a razor blade along their major axis under a stereomicroscope to increase the likelihood that chemicals would digest the mesophyll and detach both the cuticle from the underlying plant tissue and the stomata from the cuticle. Thereafter, needles of each specimen were prepared using standard laboratory treatments used for pollen analysis (including hot KOH 10%, acetolysis, and a second hot KOH 10% treatment, but excluding HCl and HF treatments (following MacDonald 2002), embedded in glycerine jelly, and mounted on slides. The number of needles prepared for each specimen varies depending on the abundance of the material that was available.

Stop-motion animations

For each specimen, we took images of flat-lying stomata at 630x magnification (Leica HI PLAN 63x objective with Koehler illumination set up) with a transmitted-light microscope (Leica DM 1000) equipped with a Leica ICC50 HD 3.1 megapixels camera. Images were taken with an open aperture diaphragm to ensure high resolution, small contrast, and small depth of view. At least 20 images were taken with the freeware Leica Acquire v.3.4.1 software at increasing focussing depths. Each image features a scale bar (0.05 mm long) that gives an estimate of stomata size. The images were z-stacked with the ImageJ v 1.50 software (Schneider et al. 2012), which provided stop-motion animations that may help the analyst to follow the stomata identification procedure.

Terminology used

Although the terminology used to describe conifer-stomata morphology was extensively described in previous publications (e.g. Trautmann 1953; Hansen 1995; MacDonald 2002), we briefly summarise the main

components of the stomata structure seen in equatorial view to provide the required vocabulary for stomata identification (Figure 2).

Conifer stomata of species analysed in this study comprise two kidney-shaped guard cells joined together at their ends, leaving an empty space between them (the stoma opening). The most distinctive guard cell feature is the presence of unevenly thickened cell walls (Evert 2006). The cell-wall thickenings formed at the junctions (the stems) extend from the stoma opening to the poles, which often terminate with polar hooks. The cell walls facing towards the outer side of the needle (the 'upper lamellae') are more lignified than the cell walls facing towards the inner side of the needle (the 'lower lamellae'). The lignified cell wall bordering the stoma opening (the 'medial lamella') is often thickened. Sometimes the upper and lower lamellae do not stay attached together. Thus, it can happen that in pollen slides the stems are attached either to the lower lamellae or to the upper lamellae.

Results

Cupressaceae

Genera: *Cupressus*, *Juniperus*

Species analysed: *Cupressus sempervirens* L., *Cupressus dupreziana* var. *atlantica* (Gaussen) Silba, *Cupressus dupreziana* A. Camus, *Juniperus communis* L., *Juniperus communis* var. *saxatilis* Pall., *Juniperus drupacea* Labill., *Juniperus oxycedrus* L., *Juniperus phoenicea* L., *Juniperus sabina* L., *Juniperus thurifera* L.

Stomata-type: *Cupressus*-type

The stomata from these taxa (Fig. 3) are characterised by relatively thick stems with arrow-tail-shaped poles. Polar hooks are absent. The medial lamellae are generally thinner than the stems and distinct. When present, the upper lamellae are distinct and almost as large as the lower lamellae. The pole-ward edges of both lamellae do not reach to the poles, and the pole-ward medial border of the upper lamellae runs close to the stem.

Pinaceae

Genus: *Abies*

Species analysed: *Abies alba* Mill, *Abies borisii-regis* Mattf., *Abies cephalonica* Loudon, *Abies cilicica* (Antoine & Kotschy) Carrière, *Abies nebrodensis* (Lojac.) Mattei, *Abies nordmanniana* Spach, *Abies nordmanniana* subsp. *equi-trojani* (Asch. & Sint. ex Boiss.) Coode & Cullen, *Abies numidica* de Lannoy ex Carrière, *Abies pinsapo* Boiss., *Abies pinsapo* var *marocana* (Trab)

Stomata-type: *Abies*-type

The stomata of the ten *Abies* taxa analysed (Fig. 4) have relatively thin stems with short and thin polar hooks, which run perpendicular to the stem. The medial lamellae are almost as thin as the stem, sometimes indistinct but generally more distinct than for *Larix* stomata. When present the upper lamellae are distinct, and are both shorter and narrower than the lower lamellae. The pole-ward medial borders of the upper lamellae are not parallel to the stem, giving the upper lamellae a butterfly-shaped form. In addition, the medial border of the upper lamellae is generally slightly concave.

Genus: *Cedrus*

Species analysed: *Cedrus atlantica* (Endl.) Manetti ex Carriere, *Cedrus libani* A. Rich.

Stomata-type: *Cedrus*-type

The two *Cedrus* species have stomata characterised by relatively thick medial lamellae that delimit a circular opening (Figs. 5a,c,e). The thickness of the stem is greatest on the upper side (Fig. 5b); towards the lower side of the stoma the stem thins out near the stoma opening (Figs. 5a,e). Polar hooks are long and generally bent (Figs. 5a,b,c,e). The upper lamellae (Fig. 5d) are as wide as the lower lamellae, but are shorter. Similarly to *Abies* stomata, the upper lamellae have a butterfly-shaped form (Figs. 5a,b,d).

Genus: *Picea*

Species: *Picea abies* (L.) H. Karst, *Picea omorika* (Pancic) Purk, *Picea orientalis* (L.) Peterm.

Stomata-type: *Picea*-type

The stomata of the three *Picea* species analysed are characterised by relatively thick and distinct medial lamellae (Figs. 6a,c,f,g). The stem thickness varies along the stoma depth: on the lower side of the stoma the stem thins out toward the stoma opening (Figs. 6a,c,f,g); on the upper side the stem has rather straight sides. The pole hooks are short, bent, distinct, and are connected to the stem by a thin junction on the lower side of the stoma (Fig. 6a,d,e). The upper lamellae are generally indistinct and are as wide and long as the lower lamellae. Their pole-ward medial border runs parallel and close to the stem. Often the stomata bear a tooth at the polar edges of the stoma opening.

Genus: *Pinus*

Species: *Pinus brutia* Ten., *Pinus cembra* L., *Pinus halepensis* Mill., *Pinus heldreichii* H. Christ, *Pinus mugo* Turra, *Pinus nigra* subsp. *laricio* (Poir.) Maire, *Pinus nigra* subsp. *salzmannii* (Dunal) Franco, *Pinus peuce* Griseb., *Pinus pinaster* Aiton, *Pinus pinea* L., *Pinus sylvestris* L., *Pinus uncinata* Ram ex DC

Stomata-type: *Pinus*-type

The stomata of these species bear thick and distinct medial lamellae and thick stems. The stems are notched more or less sharply on the lower side of the stomata (Figs. 7c,d,e,f,g,h), and are curved on the upper side (Figs. 7b,c,d,f,i,j,n). Polar hooks are generally distinct, long, bent, and are directly attached to the stem. When present, the upper lamellae are shorter and narrower than the lower lamellae.

Genus: *Larix*

Species: *Larix decidua* Mill., *Larix sibirica* Ledeb.

Stomata-type: *Larix*-type

The *Larix* stomata have relatively thin stems with long and thin polar hooks. The stem is slightly thinner on the upper side (Fig. 8a) than on the lower side (Fig. 8b). The medial lamellae are very thin (Figs. 8a,c) and usually much less distinct than in *Abies* stomata. When present the upper lamellae are indistinct, and are both shorter and narrower than the lower lamellae (Figs. 8b,d). Similarly to *Abies* and *Cedrus*, the pole-ward medial borders of the upper lamellae are not parallel to the stem. However, as opposed to *Abies*-type stomata, the medial border of the upper lamellae is generally straight (Figs. 8b,d).

Taxaceae

Genus: *Taxus*

Species: *Taxus baccata* L.

Stomata-type: *Taxus*-type

As opposed to stomata of the other genera, the stomatal complex of *Taxus baccata* includes subsidiary cells (Fig. 8e) which are located above the upper lamellae. While these are a useful feature for the identification of *Taxus baccata* stomata, often stomata are found without the subsidiary cells (Fig. 8f). *Taxus baccata* stomata have relatively thick stems with short and thin polar hooks (Fig. 8f). The medial lamellae are very thin and usually indistinct. When present the upper lamellae are very distinct, and are both shorter and narrower than the lower lamellae, and have a butterfly-shaped form. Similarly to *Abies*-type stomata, the pole-ward medial border of the upper lamellae is generally curved.

Discussion

At the level of detail achieved in our study, the stomata of European species of the Cupressaceae family show similar features among genera. The stomata of the genera *Juniperus* and of *Cupressuss* seem to be indistinguishable with simple visual examination (Fig. 3). Moreover, the stomata of these taxa have morphological traits similar to those described by Trautmann (1953) for *Juniperus sabina* and can therefore be grouped within the *Juniperus*-type.

By contrast, the stomata of European species of the Pinaceae family analysed in our study bear genus-specific features. This confirms earlier observations made by Trautmann (1953), who noted for instance that among species of *Pinus* sp. the stomata morphologies were not distinguishable with simple visual observations of stomata features. In keeping with this observation, Tonkov et al. (2018) recently grouped the stomata of *P. mugo*, *P. sylvestris* and *P. peuce* as *Pinus* sp.. The notion of genus-specific stomata morphologies has been recently thoroughly tested and confirmed using statistical analysis of 10 geometrical measurements of morphological traits in a total of 315 stomata from 10 different genera of North American conifers (Lacourse et

al. 2016). Similarly, Hu et al. (2016) and (Zhang et al. 2011) found on the basis of morphological traits that conifer species native to China could be identified to genus level.

However, other investigations reported that measurements of geometrical features might be useful to separate stomata among and within genera. For instance, Yu (1997) found different width-to-length ratios for stomata of north American and Canadian *Thuja* and *Juniperus*, two genera belonging to the Cupressaceae family. More recently García Álvarez et al. (2009, 2014) and Magyari et al. (2012) reported significant morphological differences among stomata from living populations of some European *Pinus* species on the basis of statistical analyses of morphological traits. Similarly, Hu et al. (2016) found species-specific differences of one morphological trait (the angle of attachment of the upper lamellae) among stomata of two *Abies* species native to China.

In our study we refrained from numerical analyses of size measurements and morphological traits for two main reasons. Firstly, our main goal was to provide descriptions of stomata morphologies for the 40 analysed taxa with a simple user-friendly tool that stomata analysts could refer to for stomata identification under standard transmitted-light microscopes at magnifications typically used for pollen analysis (e.g. x400 or x630). Classically, microscope analysts refer to dichotomous identification keys, static snapshot-type images of stomata, and schematic drawings to identify specimens. However, such tools only can illustrate a subset of the features of the overall morphology. Thus, for the reliable identification of stomata a good reference collection is highly recommended (MacDonald 2002). The fact that some features change with focussing cell depth was already recognised by Trautmann (1953). For instance, he pointed out that the stem thickness and shape of *Pinus* stomata varies along focus levels and therefore presented for each genus schematic drawings at two discrete focusing depths when stomata cells are observed in equatorial view. In this respect our study went some steps further because the stop-motion animations (see ESM 2-40 at http://dx.doi.org/10.6084/m9.figshare.7165261) provide a higher number of images taken at different focussing depths, thereby allowing to virtually navigate across the different layers of the stomata in order to become acquainted with their 3-dimensional structure. The importance of the 3-dimensional structure of microfossils has also been acknowledged in prior studies. For instance, pollen atlases often illustrate a series of images of pollen grains taken at different focusing depths (Reille 1992; Beug 2004). While such stop-motion animations may not replace a reference collection, an animated sequence of images taken at different focus levels can be an effective tool to visualize the changing shapes of features that are visible under the microscope (Martin and Harvey 2017). Second, the morphological variability of stomata within species and the degree of morphological overlap among species may preclude such

purely quantitative analysis (Lacourse et al. 2016). For instance, García Álvarez et al. (2009) reported for *P. sylvestris* from Spain different stomata sizes (e.g. width, length) compared to the sizes measured by Sweeney (2004) for Scandinavian specimens, indicating intraspecific variability of single morphological traits at continental scales. We collected the majority of needles from specimens living in Botanical Gardens. Because environmental conditions in Botanical Gardens represent (at best) only a fraction of the range of climatic conditions and biotopes of wild populations across Europe or even Eurasia, size measurements might be inaccurate. Conversely, because the overall stomata morphology is more stable at the species level (García Álvarez et al. 2009), our qualitative descriptions should reflect the morphologies independently of intraspecific variations. Moreover, our sampling size is rather small (only few needles from 1-2 specimens (Tab. 1)) and larger sample sizes may be needed to define quantitatively the limits of taxonomic differentiation among congeneric species (Lacourse et al. 2016).

In comparison to prior stomata-identification studies of European conifers (Trautmann 1953; Sweeney 2004) our study broadens substantially both, the regional and taxonomical coverage of this technique. Trautmann (1953) focused only on central European and Alpine conifers, and Sweeney (2004) considered only species native to Fennoscandia. Our assessment takes into account the larger variety of conifer species (and subspecies) interspersed in southern European and northern African mountains. Overall, our descriptions largely overlap with those of genus-level features that were detailed in prior studies, but some differences may be highlighted. Trautmann (1953) noted that the pole-ward medial border of the upper lamellae is straight in *A. alba* stomata and curved in *L. decidua* stomata. By contrast, we noted the opposite: a curved medial border in *Abies*-type stomata (Fig. 4) and a straighter border in *Larix*-type stomata (Fig. 8). Our description of the pole-ward medial border of the upper lamellae is however consistent with the description given by Sweeney (2004). Also, for *Pinus* sp. stomata we noted the very frequent occurrence of notched stems, a feature that was illustrated in Trautmann's schematic drawing but that received little attention in subsequent studies. Moreover, we describe for the first time the stomata morphology of *Cedrus atlantica* and *Cedrus libani*, two species having small and fragmented populations on the southern and eastern borderlands of the Mediterranean Basin. *Cedrus*-type stomata seem absent from palaeoecological records within and around the current range of these two species (Cheddadi et al. 2009; Hajar et al. 2010; Campbell et al. 2017). We cannot exclude the possibility that the stomata of these species are less resistant to decay than the stomata of European conifers. However, *Cedrus* stomata are resistant to chemical processing for pollen analyses (this study; Zhang et al. 2011). A plausible explanation for the absence of *C. atlantica* and *C. libani* stomata in palaeoecological records is that their sparse

tree cover may contribute to low abundance of needles being deposited. Moreover, the thin soil cover and seasonal aridity in their native regions (Lamb et al. 1989; Hajar et al. 2010) may cause the decay of the needles prior to their deposition in the lakes. The possibly faster decay in seasonally dry southern Europe may also provide another plausible explanation for the absence of stomata in palaeoecological records from lowland sites in southern Europe. This would fit with evidence for stomata findings in cooler and moister mountain regions (e.g. Vescovi et al. 2010; Ammann et al. 2014; Tonkov et al. 2018) than in drier and warmer lowland sites. While this may point out the limits of the method, we are confident that our descriptions be useful to take advantage of this 65-years old technique that has great potential for inferring the local presence of conifer trees and shrubs (Ammann et al. 2014).

Conclusions

In spite of the efforts made by palaeoecologists to improve the spatial coverage of pollen records in Europe during the past decades, the long-term vegetation history and its relationship to past climate and land-use changes and to changing disturbance regimes for a number of conifer species are still not well constrained. Pollen production and dispersal may vary with habitat conditions (e.g. nutrients, competition, winds) so that reconstructing the local presence of plant populations remains ambiguous if only based on presence of pollen (false presence problem, Birks and Tinner 2016). Stomata are better indicators of local presence and together with plant macrofossils may thus refine pollen-inferred reconstructions. Another limitation of pollen studies is the low taxonomical resolution that limits identification to the genus (e.g. *Picea*, or *Abies*) or sometimes subgenus level (e.g. *Pinus;* Moore et al. 1998; Beug 2004). While the descriptions of stomata morphologies provided here do not allow species-level identifications, they support (albeit qualitatively) the notion of genus-specific stomata morphologies of several conifer genera. Moreover, the novel stop-motion animations provided by our work may be useful tools for palaeoecologists who want to acquaint oneself with stomata analysis. This now 65-years old technique can substantially improve palaeoecological studies and its applications for biodiversity conservation, and for assessments of climate change and anthropogenic impacts on vegetation.

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Data availability

All stop-motion animations are publicly available at http://dx.doi.org/10.6084/m9.figshare.7165261.

Figure Captions

Figure 1: Maps illustrating the current distribution ranges of European conifer species and subspecies. Isolated populations are shown as point features (crosses, filled circles). The majority of distributions is based on shapefiles made available by Caudullo et al. (2017). However, the distribution of *Larix sibirica* is based on a shapefile developed by Malyshev (2008), the distributions of *Pinus peuce* and *Pinus heldreichii* are based on shapefiles developed within the framework of the EUFORGEN project (Vendramin et al. 2008; Alexandrov and Andonovski 2011), and the distributions of *Juniperus drupacea*, *Cedrus atlantica*, and *Cedrus libani* are based on Wazen and Fady (2016). Distributions of *Cupressus dupreziana* and *Picea orientalis* were manually digitised based on published maps (Kayacik 1955; Abdound et al. 2016). Maps were prepared within the R computing environment (R Core Team 2017).

Figure 2: Sketched drawing of conifer stomata in equatorial view (modified after Trautmann, 1953). Shading indicates level of lignification.

Figure 3: Snapshots of conifer stomata of (a) *Cupressus sempervirens* L., (b) *Cupressus dupreziana* A. Camus, (c) *Cupressus dupreziana* var. *atlantica* (Gaussen) Silba, (d) *Juniperus communis* L. (indistinct upper lamellae), and (e) *Juniperus communis* var. *saxatilis* Pall. (upper lamellae out of focus), (f) *Juniperus drupacea* Labill., (g) *Juniperus oxycedrus* L., (h) *Juniperus phoenicea* L., (i) *Juniperus sabina* L., (j) *Juniperus thurifera* L.. For the stop-motion animations see ESM 2-11, respectively.

Figure 4: Snapshots of conifer stomata (all with upper lamellae) of (a) *Abies alba* Mill, (g) *Abies borisii-regis* Mattf., (c) *Abies cephalonica* Loudon, (d) *Abies cilicica* (Antoine & Kotschy) Carrière, (e) *Abies nebrodensis* (Lojac.) Mattei, (f) *Abies nordmanniana* Spach, (g) *Abies nordmanniana* subsp. *equi-trojani* (Asch. & Sint. ex Boiss.) Coode & Cullen, (h) *Abies numidica* de Lannoy ex Carrière, and (i) *Abies pinsapo* Boiss. For the stop-motion animations see ESM 12-20, respectively.

Figure 5: Snapshots of conifer stomata of (a-d) *Cedrus atlantica* (Endl.) Manetti ex Carrière, and (e) *Cedrus libani* A. Rich. For the stop-motion animations see ESM 21-22, respectively.

Figure 6: Snapshots of conifer stomata of (a-d) *Picea abies* (L.) H. Karst, (e-f) *Picea omorika* (Pancic) Purk, and (g-h) *Picea orientalis* (L.) Peterm. For the stop-motion animations see ESM 25-27, respectively**.**

Figure 7: Snapshots of conifer stomata of (a) *Pinus brutia* Ten., (b) *Pinus cembra* L., (c) *Pinus halepensis* Mill., (d) *Pinus heldreichii* H. Christ, (e) *Pinus mugo* Turra, (f) *Pinus nigra* subsp. laricio (Poir.) Maire, (g) *Pinus nigra* subsp. *salzmannii* (Dunal) Franco, (h-i) *Pinus peuce* Griseb., (j) *Pinus pinaster* Aiton, (k) *Pinus pinea* L., (l) *Pinus sylvestris* L., (m-n) *Pinus uncinata* Ram ex DC. For the stop-motion animations see ESM 28-39, respectively**.**

Figure 8: Snapshots of conifer stomata of (a-b) *Larix sibirica* Ledeb, (c-d) *Larix decidua* Mill., and (e-f) *Taxus baccata* L.. For the stop-motion animations see ESM 23, 24, and 40.

References

Abdound F, Griffiths A, Gardner MF (2016) *Cupressus dupreziana*, from the website, Threatened Conifers of The World (http://threatenedconifers.rbge.org.uk). Last accessed 27/09/2018.

Alcamo J, Moreno JM, Nováky B, et al (2007) Europe. Climate Change 2007: Impacts, Adaptation and Vulnerability. In: Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, UK, pp 541–580

Alexandrov AH, Andonovski V (2011) EUFORGEN Technical Guidelines for genetic conservation and use of Macedonian pine (*Pinus peuce*). Bioversity International, Rome, Italy, p 6

Ammann B, Knaap WO van der, Lang G, et al (2014) The potential of stomata analysis in conifers to estimate presence of conifer trees: examples from the Alps. Veg Hist Archaeobotany 23:249–264. doi: 10.1007/s00334-014-0431-9

Beug H-J (2004) Leitfaden der Pollenbestimmung für Mitteleuropa und angrenzende Gebiete. Verlag Dr. Friedrich Pfeil, München

Birks HH, Birks HJB (2000) Future uses of pollen analysis must include plant macrofossils. J Biogeogr 27:31–35

Birks HH, Bjune AE (2010) Can we detect a west Norwegian tree line from modern samples of plant remains and pollen? Results from the DOORMAT project. Veg Hist Archaeobotany 19:325–340. doi: 10.1007/s00334-010-0256-0

Birks HJB, Tinner W (2016) Past forests of Europe. In: San-Miguel-Ayanz J, de Rigo D, Caudullo G, et al. (eds) European Atlas of Forest Tree Species. Publications Office of the European Union, Luxembourg, pp 36–39

Brewer S, Giesecke T, Davis BAS, et al (2017) Late-glacial and Holocene European pollen data. J Maps 13:921–928. doi: 10.1080/17445647.2016.1197613

Campbell JFE, Fletcher WJ, Joannin S, et al (2017) Environmental Drivers of Holocene Forest Development in the Middle Atlas, Morocco. Front Ecol Evol 5:. doi: 10.3389/fevo.2017.00113

Caudullo G, Welk E, San-Miguel-Ayanz J (2017) Chorological maps for the main European woody species. Data Brief 12:662–666. doi: 10.1016/j.dib.2017.05.007

Cheddadi R, Fady B, Francois L, et al (2009) Putative glacial refugia of *Cedrus atlantica* deduced from Quaternary pollen records and modern genetic diversity. J Biogeogr 36:1361–1371

Conedera M, Krebs P, Tinner W, et al (2004) The cultivation of *Castanea sativa* (Mill.) in Europe, from its origin to its diffusion on a continental scale. Veg Hist Archaeobotany 13:161–179

Coomes DA, Allen RB, Bentley WA, et al (2005) The hare, the tortoise and the crocodile: the ecology of angiosperm dominance, conifer persistence and fern filtering. J Ecol 93:918–935. doi: 10.1111/j.1365-2745.2005.01012.x

Evert RF (2006) Esau's Plant anatomy: meristems, cells, and tissues of the plant body: their structure, function, and development, 3rd ed. Wiley-Interscience, Hoboken, N.J.

Finsinger W, Morales-Molino C, Gałka M, et al (2017) Holocene vegetation and fire dynamics at Crveni Potok, a small mire in the Dinaric Alps (Tara National Park, Serbia). Quat Sci Rev 167:63–77. doi: 10.1016/j.quascirev.2017.04.032

Froyd CA (2005) Fossil stomata reveal early pine presence in Scotland: implications for postglacial colonization analyses. Ecology 86:579–586

García Álvarez S, Morla Juaristi C, Paull R, García-Amorena I (2014) A taxonomic tool for identifying needle remains of south-western European *Pinus* species of the Late Quaternary. Bot J Linn Soc 175:282–298

García Álvarez S, Morla Juaristi C, Solana Gutiérrez J, García-Amorena I (2009) Taxonomic differences between *Pinus sylvestris* and *P. uncinata* revealed in the stomata and cuticle characters for use in the study of fossil material. Rev Palaeobot Palynol 155:61–68. doi: 10.1016/j.revpalbo.2009.01.002

GBIF Secretariat (2017) GBIF Backbone Taxonomy. Checklist dataset. https://doi.org/10.15468/39omei. Accessed 8 Jun 2018

Gervais BR, MacDonald GM (2001) Modern pollen and stomate deposition in lake surface sediments from across the treeline on the Kola Peninsula, Russia. Rev Palaeobot Palynol 114:223–237. doi: 10.1016/S0034-6667(00)00076-2

Giesecke T, Brewer S, Finsinger W, et al (2017) Patterns and dynamics of European vegetation change over the last 15,000 years. J Biogeogr 44:1441–1456. doi: 10.1111/jbi.12974

Hajar L, François L, Khater C, et al (2010) *Cedrus libani* (A. Rich) distribution in Lebanon: Past, present and future. C R Biol 333:622–630. doi: 10.1016/j.crvi.2010.05.003

Hansen BC., Rodbell D., Seltzer G., et al (2003) Late-glacial and Holocene vegetational history from two sites in the western Cordillera of southwestern Ecuador. Palaeogeogr Palaeoclimatol Palaeoecol 194:79–108. doi: 10.1016/S0031-0182(03)00272-4

Hansen BCS (1995) Conifer stomate analysis as a paleoecological tool: an example from the Hudson Bay Lowlands. Can J Bot 73:244–252

Herring EM, Gavin DG, Dobrowski SZ, et al (2018) Ecological history of a long-lived conifer in a disjunct population. J Ecol 106:319–332. doi: 10.1111/1365-2745.12826

Hu Y-Q, Mingram J, Stebich M, Li J-F (2016) A key for the identification of conifer stomata from N.E. China based on fluorescence microscopy. Rev Palaeobot Palynol 233:12–21. doi: 10.1016/j.revpalbo.2016.06.005

Huntley B, Birks HJB (1983) An atlas of past and present pollen maps for Europe: 0-13,000 years ago. Cambridge University Press, Cambridge

IUCN (2017) The IUCN Red List of Threatened Species. Version 2017-3. <http://www.iucnredlist.org>

Kayacik H (1955) The distribution of *Picea orientalis* (L.) Carr. Kew Bull 10:481–490. doi: 10.2307/4109240

Lacourse T, Beer KW, Hoffman EH (2016) Identification of conifer stomata in pollen samples from western North America. Rev Palaeobot Palynol 232:140–150. doi: 10.1016/j.revpalbo.2016.05.005

Lamb HF, Eicher U, Switsur VR (1989) An 18,000-Year Record of Vegetation, Lake-Level and Climatic-Change from Tigalmamine, Middle Atlas, Morocco. J Biogeogr 16:65–74

Lang G (1994) Quartäre Vegetationsgeschichte Europas. Methoden und Ergebnisse. G. Fischer, Jena

MacDonald GM (2002) Conifer Stomata. In: Tracking Environmental Change Using Lake Sediments. Springer, Dordrecht, pp 33–47

Malcolm JR, Canran L, Neilson RP, et al (2006) Global warming and extinctions of endemic species from biodiversity hotspots. Conserv Biol 20:538–548

Malyshev LL (2008) AgroAtlas - Relatives - Range of distribution of Siberian Larch (Larix sibirica) . In: Afonin AN, Greene SL, Dzyubenko NI, Frolov AN (eds) Interactive Agricultural Ecological Atlas of Russia and Neighboring Countries. Economic Plants and their Diseases, Pests and Weeds [Online]. Available at: http://www.agroatlas.ru/en/content/related/Larix_sibirica/

Martin AC, Harvey WJ (2017) The Global Pollen Project: a new tool for pollen identification and the dissemination of physical reference collections. Methods Ecol Evol 8:892–897. doi: 10.1111/2041-210X.12752

Moore PD, Webb JA, Collinson ME (1998) Pollen analysis. Blackwell Science Ltd, Oxford

Orbán I, Birks HH, Vincze I, et al (2018) Treeline and timberline dynamics on the northern and southern slopes of the Retezat Mountains (Romania) during the late glacial and the Holocene. Quat Int 477:59–78. doi: 10.1016/j.quaint.2017.03.012

Parmesan C (2006) Ecological and evolutionary responses to recent climate change. Annu Rev Ecol Evol Syst 37:637–669, doi:10.1146/annurev.ecolsys.37.091305.110100

R Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

Reille M (1992) Pollen et spores d'Europe et d'Afrique du nord. Laboratoire de Botanique Historique et Palynologie, Marseille

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671–675. doi: 10.1038/nmeth.2089

Sweeney CA (2004) A key for the identification of stomata of the native conifers of Scandinavia. Rev Palaeobot Palynol 128:281–290. doi: 10.1016/S0034-6667(03)00138-6

Tinner W, Colombaroli D, Heiri O, et al (2013) The past ecology of *Abies alba* provides new perspectives on future responses of silver fir forests to global warming. Ecol Monogr 83:419–439. doi: 10.1890/12-2231.1

Tonkov S, Possnert G, Bozilova E, et al (2018) On the Holocene vegetation history of the Central Rila Mountains, Bulgaria: The palaeoecological record of peat bog Vodniza (2113 m). Rev Palaeobot Palynol 250:16–26. doi: 10.1016/j.revpalbo.2017.12.006

Trautmann W (1953) Zur Unterscheidung fossiler Spaltöffnungen der mitteleuropäischen Coniferen. Flora 140:523–533. doi: 10.1016/S0367-1615(17)31952-3

Vendramin GG, Fineschi S, Fady B (2008) EUFORGEN Technical Guidelines for genetic conservation and use for Bosnian pine (*Pinus heldreichii*). Bioversity International, Rome, Italy, p 6

Vescovi E, Ammann B, Ravazzi C, Tinner W (2010) A new Late-glacial and Holocene record of vegetation and fire history from Lago del Greppo, northern Apennines, Italy. Veg Hist Archaeobotany 19:219–233. doi: 10.1007/s00334-010-0243-5

Vincze I, Orbán I, Birks HH, et al (2017) Holocene treeline and timberline changes in the South Carpathians (Romania): Climatic and anthropogenic drivers on the southern slopes of the Retezat Mountains. The Holocene 27:1613 –1630. doi: 10.1177/0959683617702227

Wagner S, Litt T, Sánchez-Goñi M-F, Petit RJ (2015) History of *Larix decidua* Mill. (European larch) since 130 ka. Quat Sci Rev 124:224–247. doi: 10.1016/j.quascirev.2015.07.002

Wazen N, Fady B (2016) Geographic distribution of 24 major tree species in the Mediterranean and their genetic resources. Food and Agriculture Organization of the United Nations and Plan Bleu pour l'Environnement et le Développement en Méditerranée, Rome, Italy

Whittaker RJ, Fernandez-Palacios JM (2007) Island Biogeography: Ecology, Evolution, and Conservation. Oxford University Press

Yu Z (1997) Late Quaternary paleoecology of *Thuja* and *Juniperus* (Cupressaceae) at Crawford Lake, Ontario, Canada: pollen, stomata and macrofossils. Rev Palaeobot Palynol 96:241–254

Zhang K, Zhao Y, Guo XL (2011) Conifer stomata analysis in paleoecological studies on the Loess Plateau: An example from Tianchi Lake, Liupan Mountains. J Arid Environ 75:1209–1213. doi: 10.1016/j.jaridenv.2011.04.023

Figure 1a

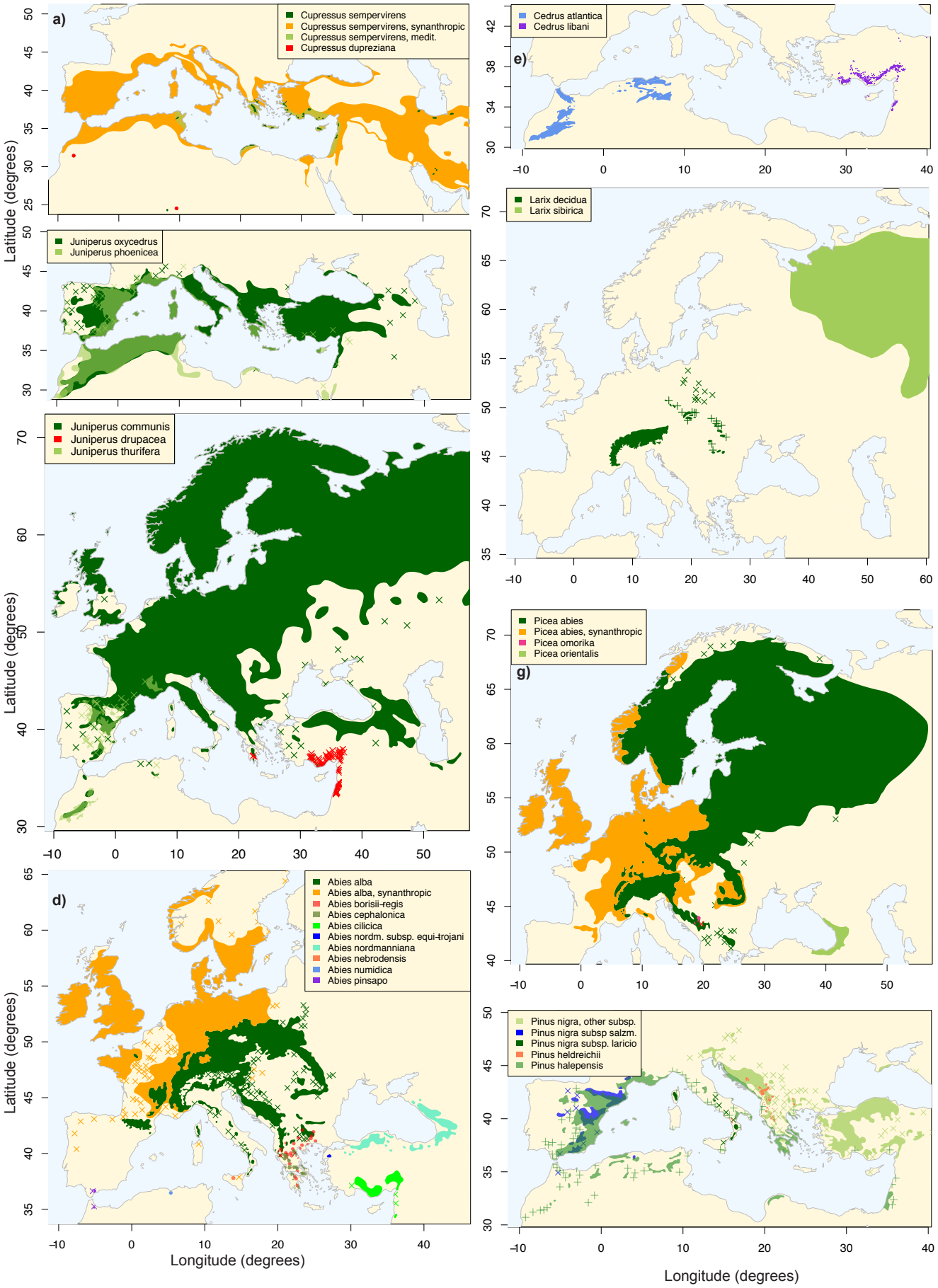

a)
Cupressus sempervirens
Cupressus sempervirens, synanthropic
Cupressus sempervirens, medit.
Cupressus dupreziana
Juniperus oxycedrus
Juniperus phoenicea
Juniperus communis
Juniperus drupacea
Juniperus thurifera
d)
Abies alba
Abies alba, synanthropic
Abies borisii-regis
Abies cephalonica
Abies cilicica
Abies nordm. subsp. equi-trojani
Abies nordmanniana
Abies nebrodensis
Abies numidica
Abies pinsapo
e)
Cedrus atlantica
Cedrus libani
Larix decidua
Larix sibirica
g)
Picea abies
Picea abies, synanthropic
Picea omorika
Picea orientalis
Pinus nigra, other subsp.
Pinus nigra subsp salzm.
Pinus nigra subsp. laricio
Pinus heldreichii
Pinus halepensis
Latitude (degrees)
Longitude (degrees)

Figure 1b

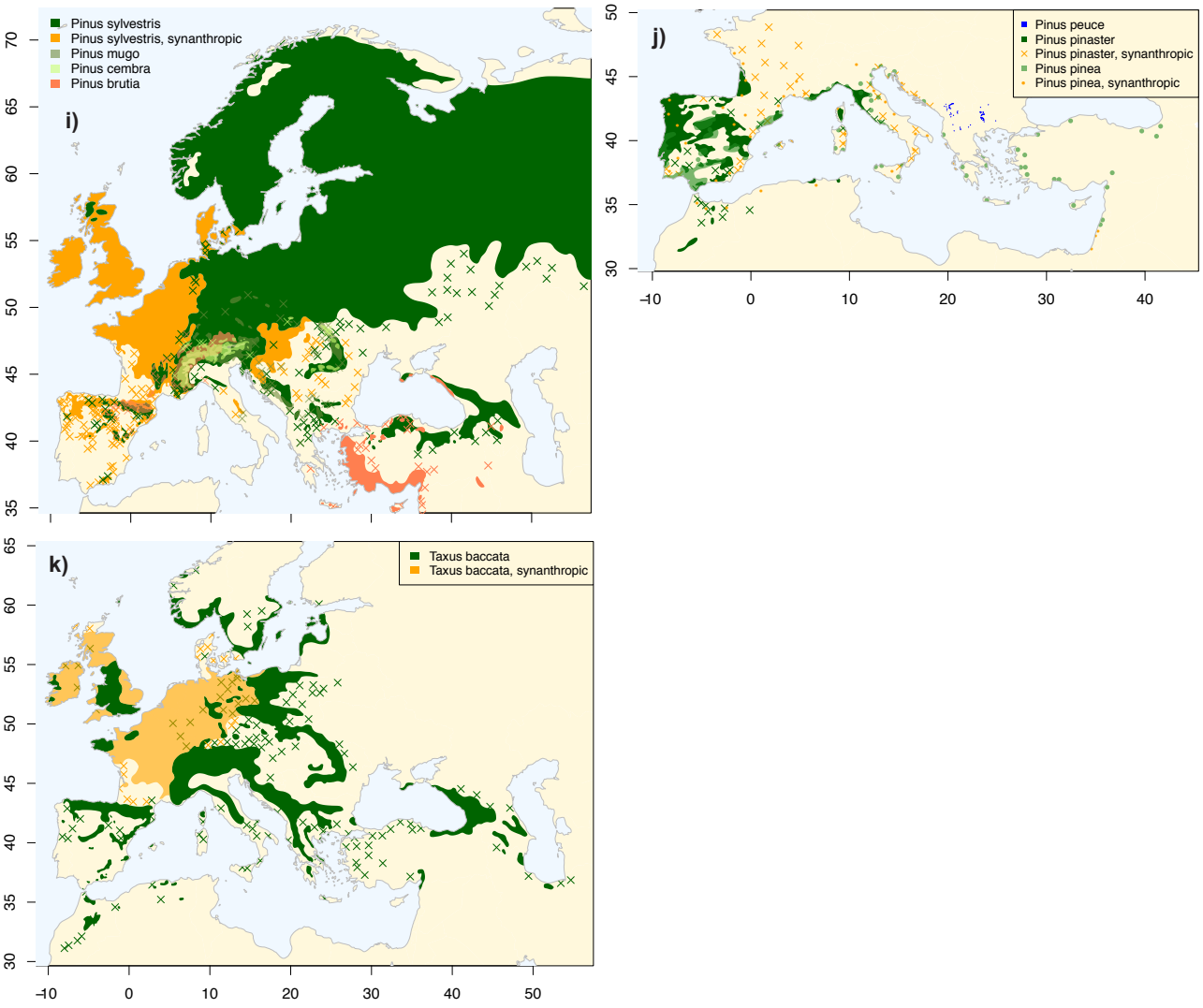
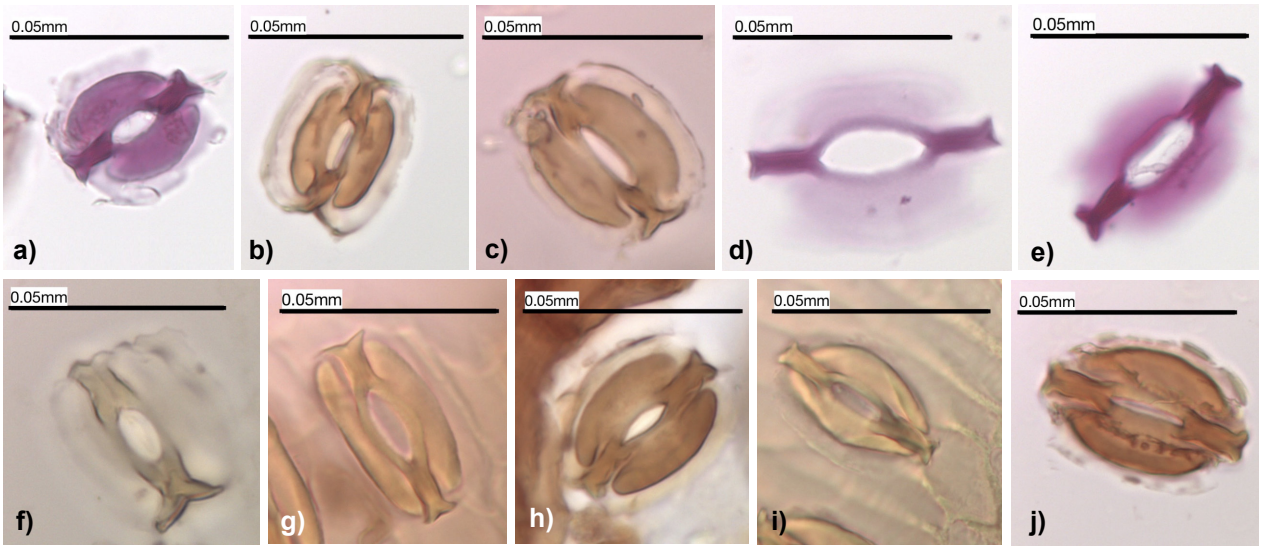

i)
Pinus sylvestris
Pinus sylvestris, synanthropic
Pinus mugo
Pinus cembra
Pinus brutia
j)
Pinus peuce
Pinus pinaster
Pinus pinaster, synanthropic
Pinus pinea
Pinus pinea, synanthropic
k)
Taxus baccata
Taxus baccata, synanthropic

Figure 3


0.05mm
a)
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f)
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g)
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h)
0.05mm
i)
0.05mm
j)

Figure 4

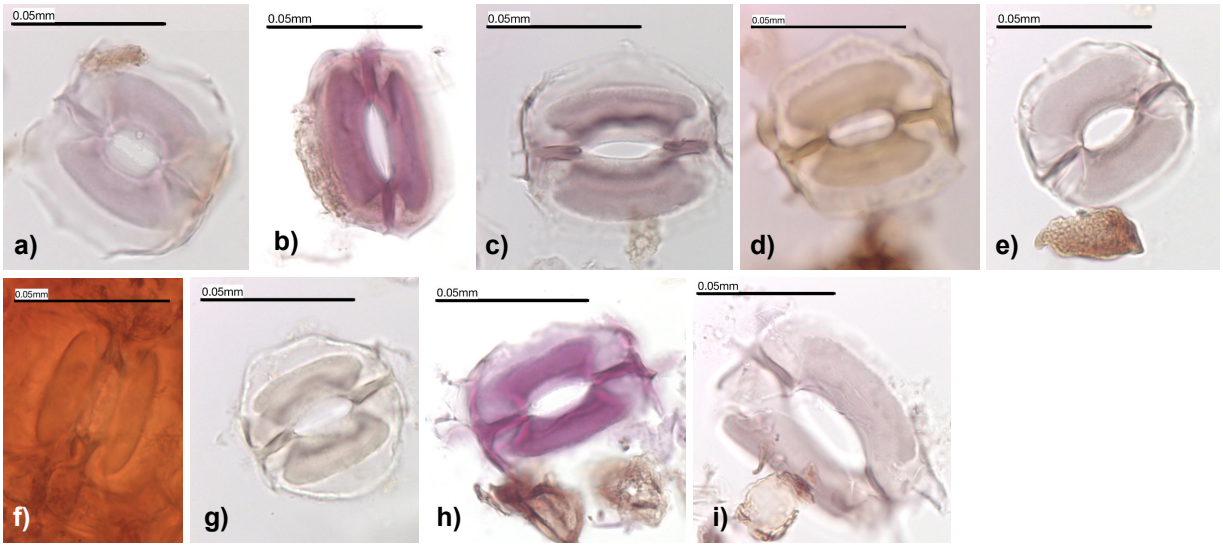
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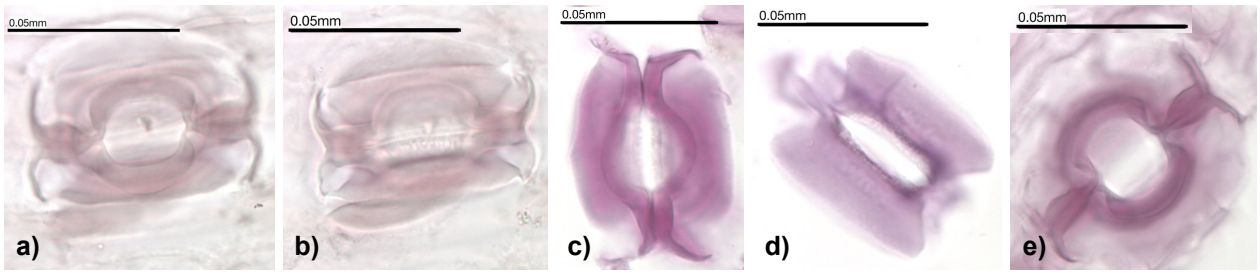
Figure 5



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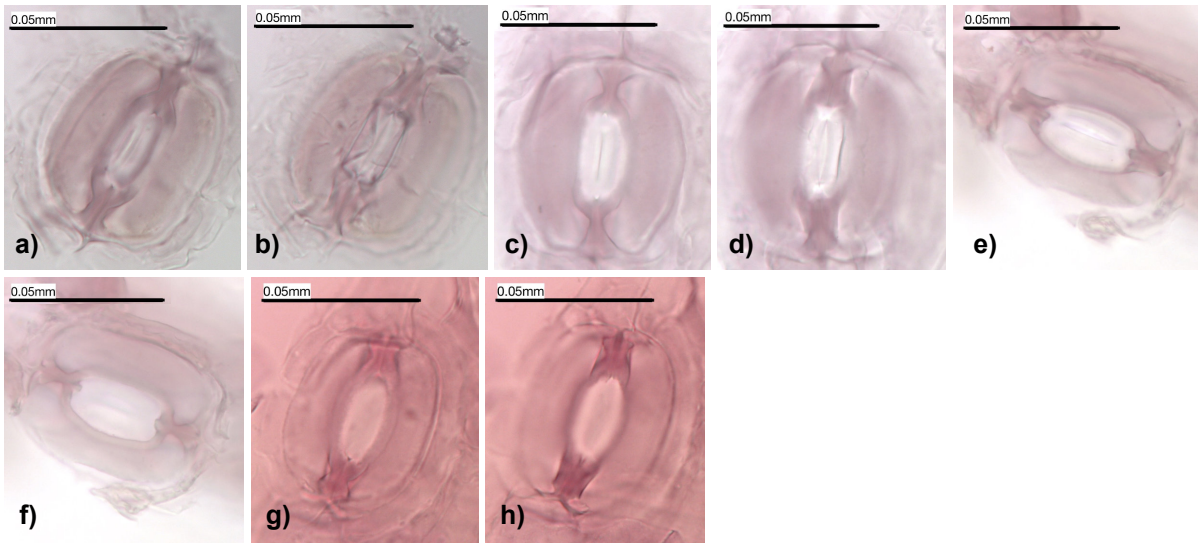

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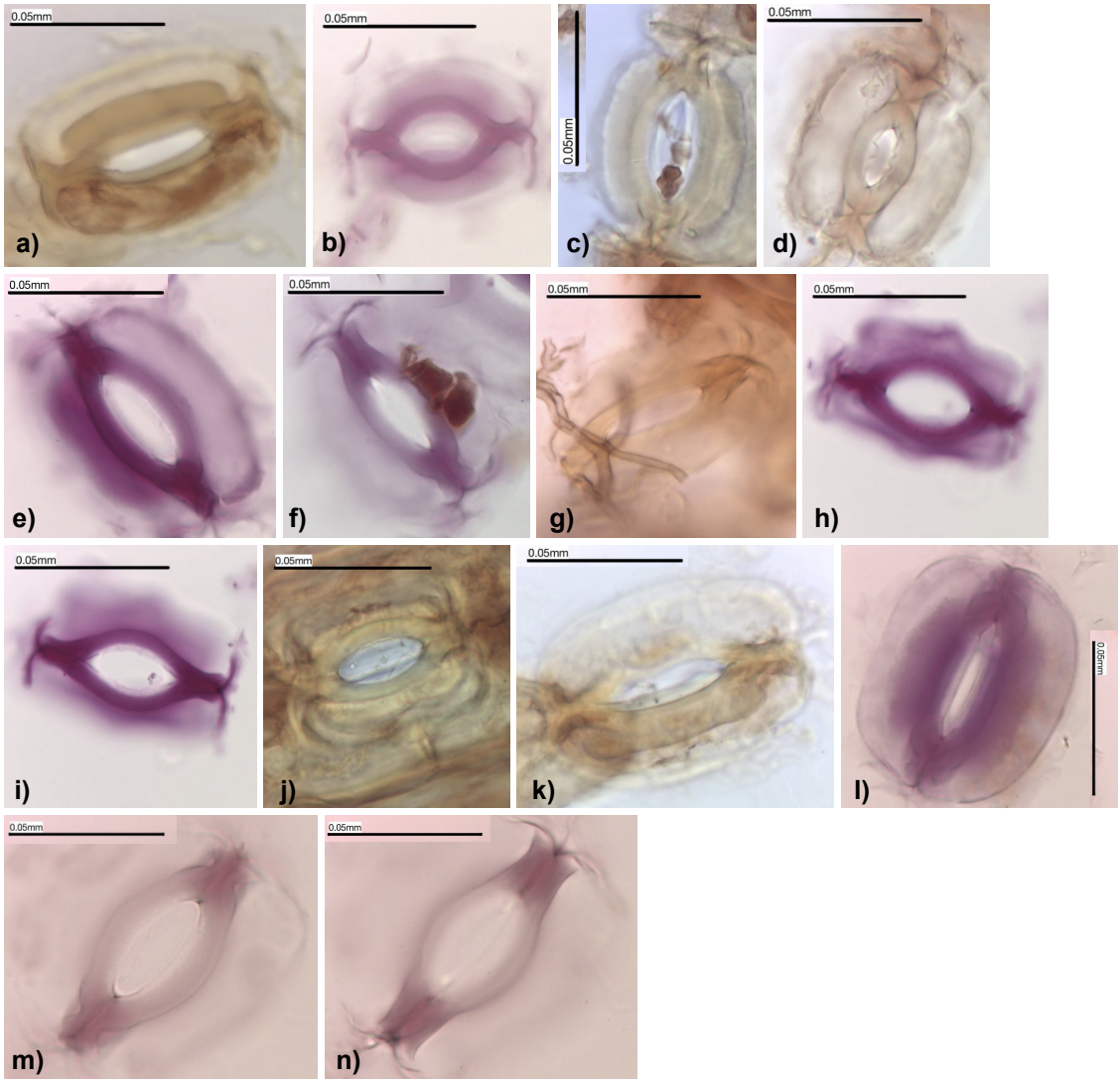

Figure 8

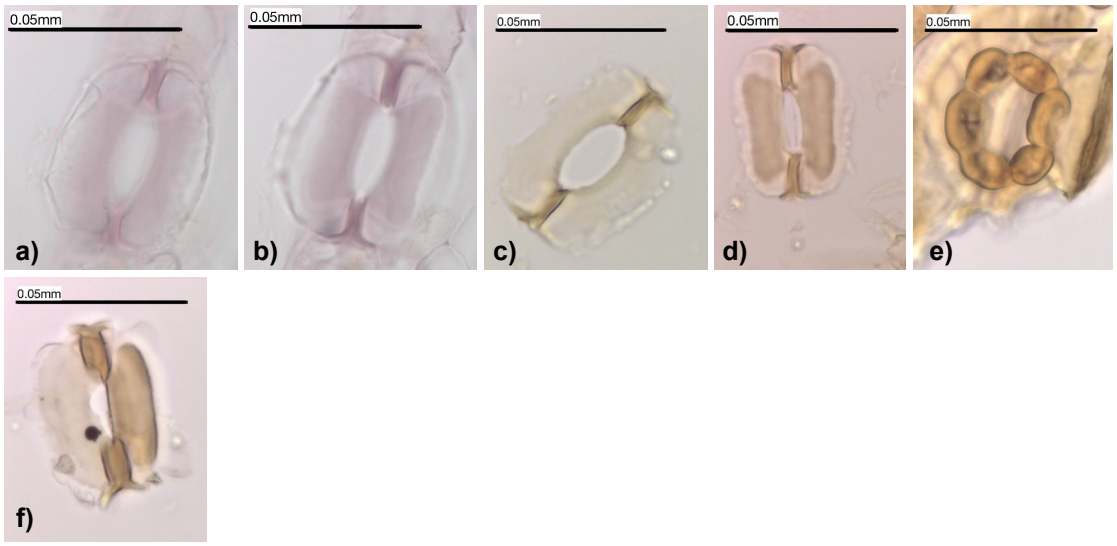

Table 1: List of species ordered by Family/Genus/Specie name following the GBIF Backbone Taxonomy (GBIF Secretariat, 2017), and number of individuals whose stomata were analyzed in this study. Abbreviations of IUCN Red List categories: least concern (LC), near threatened (NT), vulnerable (VU), endangered (EN), critically endangered (CR), not evaluated (NE) (IUCN, 2017). * = needles collected from an isotype specimen, ¶ = needles collected from a syntype.

Family	*Genus*	*Species name*	IUCN Red List Category	Stomata previously described	Number individuals in this study
Cupressaceae	*Cupressus*	*Cupressus sempervirens* L.	LC	/	1
		Cupressus dupreziana var. *atlantica* (Gaussen) Silba	CR	/	1
		Cupressus dupreziana A. Camus	EN	/	1
	Juniperus	*Juniperus communis* L.	LC	Trautmann (1953) Sweeney (2004)	1
		Juniperus communis var. *saxatilis* Pall.	LC	Trautmann (1953)	1
		Juniperus drupacea Labill.	LC	/	1
		Juniperus oxycedrus L.	LC	/	1
		Juniperus phoenicea L.	LC	/	1
		Juniperus sabina L.	LC	Trautmann (1953)	1
		Juniperus thurifera L.	LC	/	1
Pinaeceae	*Abies*	*Abies alba* Mill	LC	Trautmann (1953) Sweeney (2004)	1
		Abies borisii-regis Mattf.	NE	/	1
		Abies cephalonica Loudon	LC	/	1
		Abies cilicica (Antoine & Kotschy) Carrière¶	NT	/	1
		Abies nebrodensis (Lojac.) Mattei	CR	/	1
		Abies nordmanniana Spach	LC	/	1
		Abies nordmanniana subsp *equi-trojani* (Asch. & Sint. ex Boiss.) Coode & Cullen*	EN	/	2
		Abies numidica de Lannoy ex Carrière	CR	/	1
		Abies pinsapo Boiss.	EN	/	1
		Abies pinsapo var *marocana* (Trab)	EN	/	1
	Cedrus	*Cedrus atlantica* (Endl.) Manetti ex Carriere	EN	/	1
		Cedrus libani A. Rich.	VU	/	1
	Larix	*Larix decidua* Mill.	LC	Trautmann (1953)	1
		Larix sibirica Ledeb.	LC	Sweeney (2004)	1

Pinaceae	*Picea*	*Picea abies* (L.) H. Karst	LC	Trautmann (1953) Sweeney (2004)	2
		Picea omorika (Pancic) Purk	EN	/	3
		Picea orientalis (L.) Peterm.	LC	/	1
	Pinus	*Pinus brutia* Ten.	LC	/	1
		Pinus cembra L.	LC	Trautmann (1953)	2
		Pinus halepensis Mill.	LC	/	1
		Pinus heldreichii H. Christ	LC	/	2
		Pinus mugo Turra	LC	Trautmann (1953)	2
		Pinus nigra subsp. *laricio* (Poir.) Maire	LC	/	2
		Pinus nigra subsp. *salzmannii* (Dunal) Franco	LC	/	1
		Pinus peuce Griseb.	NT	/	1
		Pinus pinaster Aiton	LC	/	1
		Pinus pinea L.	LC	/	1
		Pinus sylvestris L.	LC	Trautmann (1953) Sweeney (2004)	1
		Pinus uncinata Ram ex DC	LC	/	1
Taxaceae	*Taxus*	*Taxus baccata* L.	LC	Trautmann (1953) Sweeney (2004)	1